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Historical perspective

The enzymatic sphingomyelin to ceramide conversion increases the shear membrane viscosity at the air-water interface

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ABSTRACT

Whereas most of lipids have viscous properties and they do not have significant elastic features, ceramides behave as very rigid solid assemblies, displaying viscoelastic behaviour at physiological temperatures. The present review addresses the surface rheology of lipid binary mixtures made of sphingomyelin and ceramide. However, ceramide is formed by the enzymatic cleavage of sphingomyelin in cell plasma membranes. The consequences of the enzymatically-driven ceramide formation involve mechanical alterations of the embedding membrane. Here, an increase on surface shear viscosity was evidenced upon enzymatic incubation of sphingomyelin monolayers. The overall rheological data are discussed in terms of the current knowledge of the thermotropic behaviour of ceramide-containing model membranes.

1. Introduction

From the seminal paper of Singer and Nicholson [1], cell membranes were postulated to be two-dimensional fluid structures. Through hydrophobic and hydrophilic interactions, the lipid bilayer structure could thus accommodate other biomolecules such as integral membrane proteins. Lipid fluidity was therefore invoked to be the dynamic property responsible for protein translational diffusion within the membrane. Moreover, functional membranes were conceived in terms of the lipid viscosity and changes in membrane fluidity, such as it would be triggered by changes in temperature or by different compositions of membrane phospholipids, might lead to membrane dysfunction. For long time, cell membranes were hence considered to be a short –ranged oriented solution of interacting integral proteins embedded in a continuous viscous phospholipid bilayer solvent.

Very early, thermotropic transitions from a gel (Lβ, ordered-chain) state to a liquid disordered (Lα, disordered-chain) state of lipid bilayers was detected for saturated phospholipids such as the canonical dipalmitoylphosphatidylcholine (DPPC) [2]. It was already established that the temperature for the gel to liquid transition (T_m or melting temperature) depends largely on the chain-length and unsaturation of the acyl chains and on the chemistry of the polar head-group [3]. Thus, DPPC undergoes a phase transition at approximately 42 °C. Saturated phospholipids with longer acyl chains have indeed higher T_m. Remarkably, biological membranes are preserved fluid at physiological temperatures thanks to the presence of membrane proteins and Chol [4].

Simons and Ikonen revisited in 1990s the classical picture of the fluid plasma membrane. The idea that the plasma membrane could be marbled with smaller and more ordered domains able to laterally move within the lipid bilayer originated the so-called “raft hypothesis” [5]. Based on the particular interactions that govern the sphingolipid and cholesterol (Chol) interactions, lipid rafts were hypothesized to be enriched in sphingomyelin (SM) and chol, originating the denominated liquid ordered (l_o) phase [6]. In terms of viscous flowing, the l_o phase behaves as an intermediate state between Lα and Lβ phases. Although lipid rafts were attributed to play an important role in many cellular processes their existence of l_o phases in biological membranes remains still controversial [7].

However, the role played by solid phases in biological membranes has been recently revisited as the existence of ceramide (Cer)-enriched domains has been reported in different metabolic and cellular events as cell proliferation, apoptosis or disease [8,9,10]. The biological function of Cer-enriched domains could depend on the alterations of membrane biophysical properties [11] that occur upon Cer enzymatic formation [12,13,14,15]. Besides the *de novo* synthesis pathway of ceramides in the endoplasmic reticulum [16], ceramides can be generated in the plasma membrane through the action of the enzyme sphingomyelinase

Abbreviations: bCer, brain ceramide; bSM, brain sphingomyelin; Cer, Ceramide; SM, Sphingomyelin; eggCer, egg ceramide; eggSM, egg sphingomyelin; pSM, palmitoyl sphingomyeline; pCer, palmitoyl ceramide; SMase, sphingomyelinase

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(SMase) that hydrolyses the SM to phosphocholine and Cer [17]. The Cer levels in the plasma membrane are extremely low but can rapidly increase by the action of SMase in response to different stimuli [18], eliciting a number of different biological responses [8,9,10]. Accordingly, the binary mixture SM + Cer is the structural basis of Cer-enriched domains in cell membranes. Thus, the alterations of the membrane might be in part related to the unique viscoelastic properties of ceramides. Whereas most of lipids have viscous properties and they do not have significant elastic properties, ceramides behave as very rigid solid assemblies, displaying viscoelastic features at physiological temperatures [19]. Several rheological studies have been addressed to understand the physico-chemical interactions between SM and Cer and their implications in the Cer-induced alteration of the viscoelastic membrane properties.

In this review we first summarize the relevant information obtained by compression and shear surface rheology of premixed SM/Cer monolayers (Sections 2 and 3). We then present new data describing the flowing properties of SM monolayers upon SMase-driven conversion to Cer (Section 4), emphasizing the correlation between the topographical phase information of SM/Cer monolayers (Section 5) with the mechanical perturbations associated to enzymatic SM to Cer conversion (Section 6). Finally, we discuss the role played of the viscoelastic alterations by the SM to Cer conversion in modulating the mechanical and physical properties of model bilayers composed of more complex mixtures.

2. Π-A compression isotherms of SM/Cer mixtures

The monolayer isotherms of mixtures of SM/Cer at different proportions have been studied earlier for different alkyl species [20,21,22]. A liquid-expanded (LE, chain-disordered) to liquid-condensed (LC, chain-ordered) phase transition is found for intermediate chain SMs (from C14:0 to C26:0, and C24:1) at low lateral pressures. The lateral pressure of the two-dimensional transition clearly depends on the acyl composition of SMs and the temperature. The lower the temperature and the longer the acyl chain, then the lower the surface pressure (and larger the molecular area) that are observed at the onset of the two-dimensional phase transition [23]. A different surface behaviour has been found for unsaturated (C18:1) and very long chain polyunsaturated SMs. For this case, the compression isotherms were compatible with a LE state and showed large molecular areas [24]. In contrast, a LE behaviour, is observed for short 12:0 SM at all temperatures in the 10–30 °C range without any indication of two-dimensional phase transitions [23].

Air/water monolayers of natural ceramides present a continuous isotherm in the whole range of surface pressures at room temperatures. For larger molecular areas, isotherms remain at a near-zero pressure that is compatible with a diluted state. Upon compression, the isotherms suddenly rise, eventually entering a collapse regime, characterized by a constant pressure. Generally, the isotherms display a condensed-like shape characterized by a high slope, typical of solid phases [20,21]. Synthetic ceramides such as pCer can exhibit expanded, condensed and solid states. This polymorphic behaviour depends on the temperature and reflected an accurate phase diagram [25]. A similar multiphasic behaviour has been found for natural ceramides [26] and mixed (short and longer) ceramides [27].

The addition of increasing amounts of Cer generally produces a condensing effect on SM monolayers. As an example, Fig. 1 shows the isotherms obtained for mixtures of pSM/pCer in different proportion. In this case, the LE-LC phase transition of SM occurred at 25 mN/m and the condensing effect of pSM by the presence of pCer was more pronounced at lower lateral pressures [20]. A similar condensing effect has been reported for eggSM/eggCer monolayers [21].

The compression response is mainly driven by membrane compactness, *i.e.* a high compression modulus describes the ability of lipids to densely pack in such a way that molecular hard cores resist against

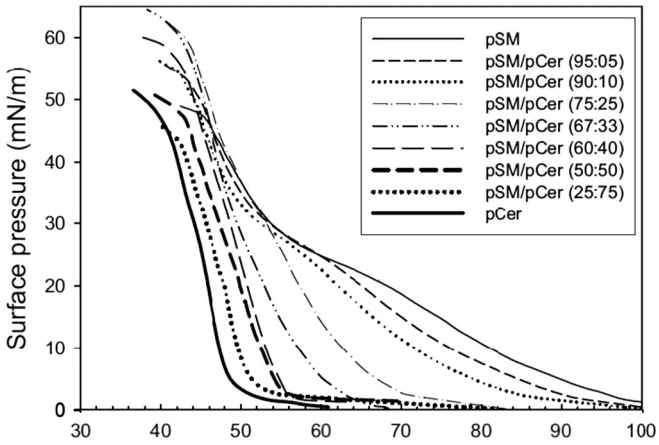

Fig. 1. Compression isotherms of pSM-pCer mixed monolayers: pure pSM (thin solid line), pure pCer (thick solid line), and pSM-pCer mixtures at 5 mol% (thin short-dashed line), 10 mol% (thin dotted line), 25 mol% (thin dot-dashed line), 33 mol% (thin double-dot-dashed line), 40 mol% (thin long-dashed line), 50 mol% (thick short-dashed line), and 75 mol% (thick dotted line) of pCer.
Figure adapted from [20]. Copyright permissions in progress.

further compression. The equilibrium compression modulus C^{-1} can be easily calculated from the numerical derivative of the experimental π-A isotherms as

$$C^{-1} = -A\frac{\partial \pi}{\partial A}$$

As expected from the isotherm curves, ceramides are characterized by a much higher modulus ($C_{Cer}{}^{-1} \approx 300$ mN/m for natural bCer, eggCer or pCer) than SMs ($C_{SM}{}^{-1} \approx 100$ mN/m, for natural bCer, eggCer or pCer) at lateral pressures ranging from 25 to 35 mN/m, corresponding to the biologically relevant surface packing [28]. Even higher values of the compression modulus for other synthetic ceramides have also been reported (400 mN/m and 600 mN/m for C24:1Cer and C16Cer respectively) [29]. Fig. 2 shows the compression modulus of eggSM/eggCer mixtures [21]. Note that the addition of increasing amounts of ceramides raises the compression modulus on SM monolayers, a fact related to the condensation effect induced by ceramides.

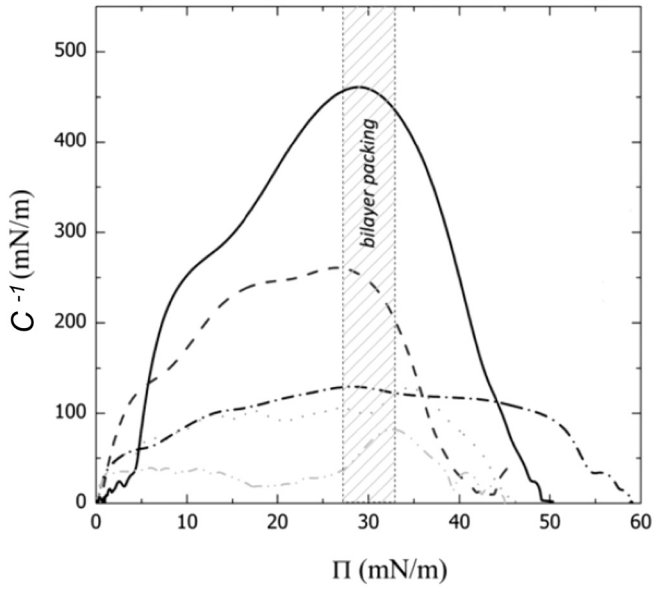

Fig. 2. π-Dependence of the compression modulus, C^{-1}, for lipid monolayers made of eggSM/eggCer mixtures: (solid line) 1:0 mol; (dashed line) 2:1 mol; (dash-dotted line) 1:1 mol; (dotted line) 1:2 mol; and (dashdot- dot-dashed line) 0:1 mol).
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The rigidifying effect was also found in binary mixtures made of different species of SM and ceramides [22].

3. Shear viscoelasticity of SM/Cer mixtures

Although the compression response has been studied in detail for lipid monolayers, and in particular for SM/Cer mixtures, little or no attention has been addressed to the shear rheology of lipid monolayers [19,21,26,30,31]. The characterization of the interfacial shear and loss moduli (G' and G'' respectively) provides valuable information about the viscoelastic character of lipid monolayers. In fact, the shear modulus measures the resistance of a structural arrangement of molecules to be distorted against a lateral shear deformation without changing the area per molecule but affecting the relative orientation between them. Consequently, solid membranes exhibit structural rigidity characterized by a finite shear modulus ($G' > 0$), independent on the compactness of the molecular packing. Moreover, fluid membranes are characterized by more or less high values of the loss modulus (which is related to the shear viscosity by $\eta = \frac{G''}{\omega}$ in oscillatory motion) and by a zero shear modulus, which defines a system able to flow under an applied force. From the pioneer work of Espinosa et al. [19], a large variety of mechanical behaviour under lateral shear flow was evidenced for different lipid species and mixtures.

In particular the viscoelastic character of eggSM/eggCer mixtures has been recently reported in Langmuir monolayers [21]. For the biologically relevant state (π = 30 mN/m), Langmuir monolayers of eggCer were characterized by a high shear modulus (G' = 80–100 mN/m) (Fig. 3A), a value three orders of magnitude higher than that measured for DPPC monolayers at gel state [19]. It has been also reported that Cer monolayers undergo plastic deformations in response to an applied stress exceeding the yield point, and this is revealed by a stress softening observed upon faster compression rates at increasing deformation [31]. Although increasing eggSM contents decreased the solid character of eggCer monolayers, G' remained finite up to a relatively high content of eggSM ($G' > 0$ for $X_{Cer} > 33\%$), indicating the high capacity of Cer to impart solid order to SM-based membranes. EggSM monolayers alone were characterized by a zero shear modulus, an evidence for a liquid state.

Flow properties are remarkably different for Cer monolayers as compared to its parent molecule SM. The surface viscosities of solid Cer monolayers display values as high as η = 200 mN s/m (at a reference frequency, ω = 0.1 s^{-1}, Fig. 3B). In contrast, the monolayers of eggSM indeed show a high fluidity, characterized by surface viscosities as low as η = 0.1 mN s/m (Fig. 3) (at ω = 0.1 s^{-1}). Mixing Cer with higher SM contents elicits a progressive increase in fluidity (Fig. 3C), which occurs in parallel to the decrease in solid character imposed by Cer. The inversion between the solid-like and a fluid-like behaviour occurred close to a 2:1 eggSM/eggCer compositional ratio. Higher proportions of eggSM are characterized by a relatively low solid character ($G'' > G' \neq 0$) and a high fluidity, typical of eggSM.

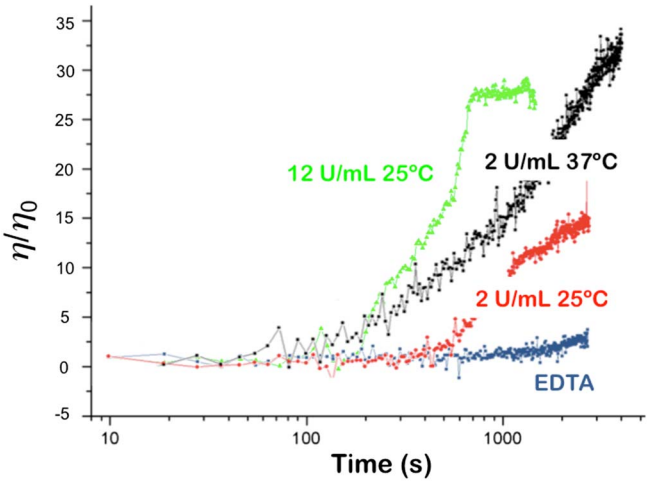

Fig. 4. Real time course of shear viscosity obtained for eggSM monolayers (π = 20 mN/m) upon SMase incubation under oscillatory shear (1% strain and at constant frequency of 1 Hz) and at different conditions.

4. Shear viscoelasticity of SM to Cer enzymatic conversion

Together with the compression studies of SM/Cer mixtures, the recent studies on their shear rheology provide an accurate picture of the mechanical behaviour of SM/Cer monolayers at the air-water interface at equilibrium. The mechanical perturbations undergone by SM monolayers upon SMase enzymatic conversion have not been addressed so far. The question about how the shear viscoelasticity of SM monolayers is affected by it enzymatic conversion raises.

Fig. 4 shows the real time course of shear viscosity obtained for eggSM monolayers (π = 20 mN/m) upon SMase incubation under oscillatory shear (1% strain and at constant frequency of 1 Hz). Independent of the temperature and the enzyme concentration, the surface viscosity generally increases after a lag time, up to a steady value. This behaviour is compatible with the enzymatic activity exhibited by SMase during degradation of bSM in the LE state (π = 10–15 mN/m), described by Fanani and Maggio [32]. These authors showed a lag time before SMase reaches its maximum activity [32]. After the lag time, the enzymatic activity reaches a steady-state regime, subsequently followed by a gradual halting of product formation.

The incubation of SMase at low concentrations (2 U/mL) resulted in a 15-fold increase of the surface viscosity. The incubation at higher amounts of SMase (12 U/mL) increased the surface viscosity of SM monolayers up to 30-fold. An analogous effect was also observed when

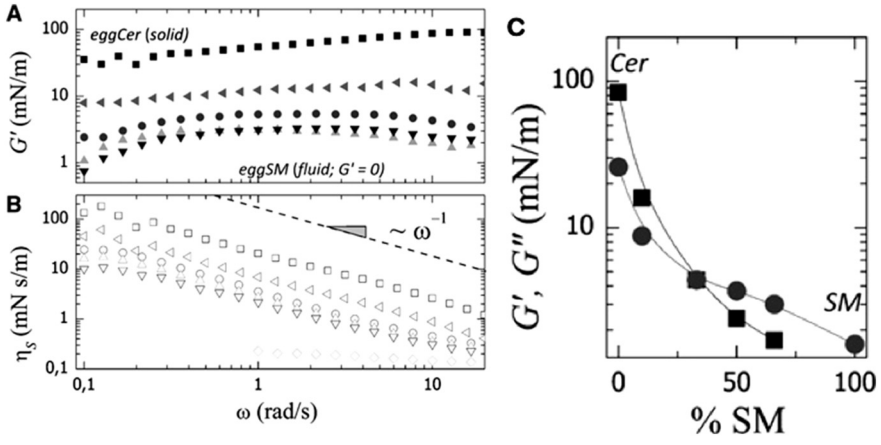

Fig. 3. (A) Frequency dependence of the shear modulus G' and (B) the shear viscosity η obtained from oscillatory shear experiments at a fixed strain amplitude of 0.5% for different eggSM/eggCer monolayers: (■) 1:0 mol; (◄) 9:1 mol; (●) 2:1 mol; (▲) 1:1 mol; (▼) 1:2 mol; and (♦) 0:1 mol. (C) Shear modulus G' (■) and the frictional shear losses G″(●) measured at a fixed frequency of 10 Hz and represented as a function of the SM content present in the same eggSM/eggCer mixtures shown in panels A and B.
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the subphase is warmed up to $T = 37$ °C probably due to a change from the LC to the LE state of the SM monolayers. This increase of the surface viscosity was prevented by the addition of EDTA in the subphase. EDTA is a chelating agent that binds to Ca^{2+} and Mg^{2+} and prevents the enzymatic activity of SMase [17].

Remarkably, the shear modulus did not exhibit a finite value upon SMase incubation ($G'(t) = 0$). This feature is a clear distinctive of the fluid state of these monolayers. Note that Fanani et al. reported a 50 mol% of SM to Cer conversion after 3 min of enzymatic activity in bSM monolayers and upon incubation with similar protein concentrations [33]. Interestingly, even at lower Cer concentrations (down to 33% mol) in premixed eggCer/eggSM monolayers a very low, but still a finite, shear rigidity ($G' \neq 0$) is promoted, although the lipid mixtures essentially behave as fluid monolayers ($G'' > G'$) [21]. Consequently, the Cer-enriched monolayers should be expected, through the action of SMase, to be much more solid and viscous than the bare SM monolayers.

5. Interfacial phase behaviour of SM monolayers under enzymatic conversion to Cer

To explain the apparent inconsistency between the solid character of premixed SM/Cer monolayers and the fluid behaviour found in enzymatically produced SM/Cer monolayers, we must invoke the different two alternative ways of enriching the Cer content of a SM monolayer: either by premixing increasing amounts of Cer in the lipid composition, or by locally increasing the amount of Cer by the enzymatic transformation of SM. In the first case, the Langmuir monolayers are in equilibrium whereas the local perturbations that are induced by the sudden interconversion of the phospholipid to Cer is a non-equilibrium process that can trigger different dynamic effects.

The enzymatic bSM to bCer conversion leads to the formation of lateral Cer-enriched domains in bSM monolayers. After the first fluorimetric evidences of phase separation induced by SMase [34], the direct visual evidence in real-time for SMAse-induced formation of Cer-enriched domains in SM monolayers was obtained by epifluorecence microscopy [33]. The premixed interface generally contains significantly larger but fewer domains and percolation occurred at lower content of Cer [33,35] (Fig. 5). Similar effects have been reported for pSM/pCer monolayers [24]. However, the enzymatically generated pSM/pCer interfaces show long-range domain ordering into a hexagonal lattice because the interdomain repulsion overcomes the thermal energy. A more detailed image analysis [36,37] revealed that the SM to Cer formation was mediated by a spontaneous nucleation of circular domains that grow in time. Circular shaped domains were transformed into flower-like domains, which eventually formed the hexagonal lattice. The formation of these domain super-structures is specific for the SMase-driven system since they could not be observed in mixed SM/Cer monolayers in the absence of SMase.

6. SMase-driven SM/Cer monolayers as a composite material

The topographical information depicts SMase-driven SM/Cer monolayers as a composite material made of Cer-enriched solid domains spread on a continuous SM-phase. Form the mechanical point of view one could expect that the presence of solid Cer-enriched domains within the fluid phase could increase the membrane rigidity either under shear or compression. However, the rheological characterization is compatible with a macroscopic fluid with microscopic condensed domains coexisting within the fluid phase characterized by vanishing shear moduli but supporting compression deformations. This can be explained by the fact that the condensed domains produced by the action of the SMase are not connected due to their interdomain repulsive interaction and the system can flow through fluid 2D channels made of the continuous fluid phase (Fig. 6A) [38,39]. The shear viscosity increase upon SMase incubation is caused by the increasing

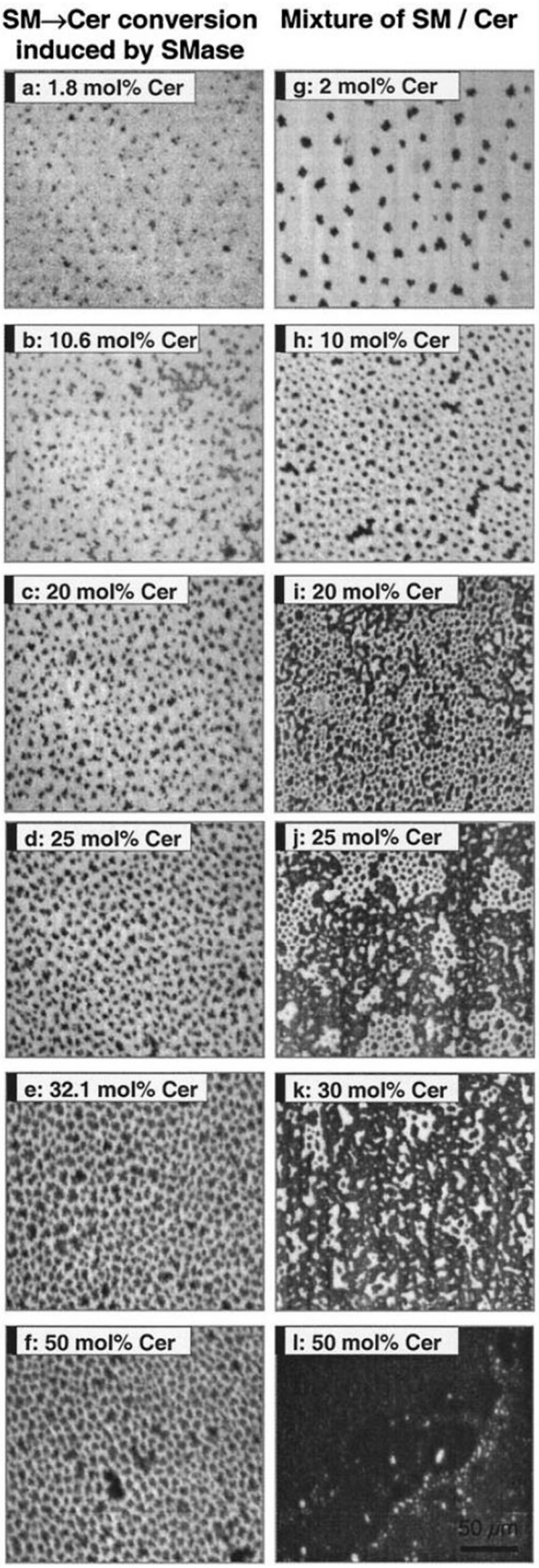

Fig. 5. Surface topografy of SM/Cer monolayers during SMase-driven conversion (left column) or previously mixed at different molar ratios (right column).
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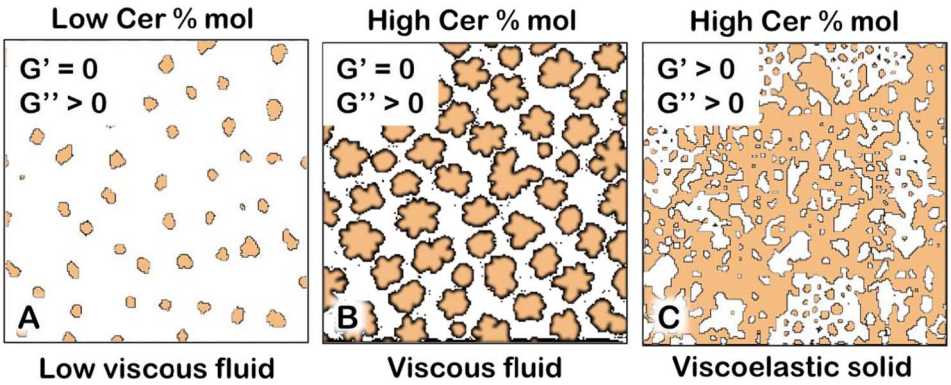

Fig. 6. Shear properties of SM/Cer monolayers upon sphingomyelinase incubation (A and B) and as premixed (C).
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amounts of the newly formed ceramides, partitioning in the continuous phase leading to the higher intrinsic viscosity and domain crowding [40]. At very high domain crowding, the monolayer viscosity increases because the domains are held in place by steric hindrance generated by the other condensed domains of the array [40] (Fig. 6B).

In contrast, the interconnected Cer-enriched domains in premixed monolayers provide a solid continuous phase able to resist shear deformation ($G' > 0$) (Fig. 6C). As Cer-enriched phase percolation seems to be a condition for the monolayer solidification, further work need to be performed with other SM species such as 28:4 SM/28:4 SM. The lower interdomain repulsion of the newly formed 28:4 SM/28:4 Cer would favour the fusion of Cer-enriched domains eventually leading to percolation [24] and thus to solid monolayers upon the action of SMase.

7. Discussion

The plasma membrane composition comprises multiple lipids and proteins. The accurate understanding the rheological alterations of the SM to Cer conversion requires the use of more complex systems that include other important lipid species as Chol or phospholipids. The presence of POPC and Chol in addition to SM affects the SMase activity is two ways. On one hand, the SMase depends critically on the phase state of the membrane as it is preferably located in expanded phases [41] as the increasing of condensed phases decrease the protein activity [42,43]. In addition, the existence of phase boundaries composed of l_o/l_d phases enhances also the enzymatic activity due to a favourable lateral diffusion of the substrate or the product in monolayers [42] or to the rapid flip-flop of Cer leading to an enhanced availability of the substrate in bilayers [44,45,46].

This is compatible with the fact that Chol interacts with SM promoting a higher local concentrations of the substrate for enzymatic conversion. Note that both SM/Chol and POPC/SM/Chol monolayers behave as very low viscous fluids [19]. Lipid mixing and the presence of lateral domains increase fluidity due to the induced molecular disorder and lubrication effects respectively. On the other hand, there is a feedback regulation of SMase activity by the Cer-induced condensation [42,33]. Not only the new-formed condensed phase decrease the activity rate of SMase but the percolation of the condensed phase prevents the protein activity in premixed SM/Cer monolayers [33] that leads to solid phases ($G' > 0$) (Fig. 6C) and might impede the Brownian motion of SMase for optimal function.

Finally, the proportions of ordered and disordered domain coexistence, ultimately given by the Chol concentration, result in a different incorporation of Cer after SM hydrolysis. Generally, a high concentration of Chol promote Cer dilution into the l_o domains whereas a low concentration of Chol enables the formation of Cer-enriched domains [47,48,49]. A similar effect has been reported with different SM/Cer molar ratios. At high SM contents, SMase induces the macroscopic Cer-enriched domains, whereas at low SM contents, solid Cer domains appear and abolish the SMAse activity [50]. To establish the rheological implications on these effects further work is required.

Although multiple factors such as lipid complexity, membrane proteins, curvature or lipid asymmetry must be considered when trying to generalize the observations made on lipid model systems to cell membranes, the rheological studies of sphingolipids-based monolayers and the SMase enzyme give a reliable information to understand the mechanical membrane alterations promoted by the enzymatic SM to Cer conversion and their interplay with the cellular processes in which Cer are involved.

8. Conclusions

Lipid binary mixtures made of SM and Cer display a rich surface topography and viscoelastic rheology and they are usually considered as solid membranes due to the unique biophysical properties of ceramides. However, Cer is formed by the enzymatic cleavage of sphingomyelin in cell plasma membranes and the membrane alterations driven by the SM conversion cannot be inferred from the equilibrium properties found in premixed membranes. Here, we have shown that the conversion of SM to Cer upon enzymatic incubation leads to an increase in the shear surface viscosity of SM monolayers. In contrast, any solid character was detected upon enzymatic conversion. This rheological behaviour is compatible with a macroscopic fluid phase coexisting with microscopic condensed domains. Unlike premixed membranes, the repulsive interdomain interactions prevent the coalescence and percolation of the newly Cer-enriched domains, thus the membrane is mechanically characterized by a vanishing shear moduli and a high viscosity. To conclude, the mesoscale phase structure has an impact not only on the enzymatic activity displayed by SMase but on the mechanical membrane alterations promoted by the protein activity.

Acknowledgements

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